

AD_____

Award Number: W81XWH-04-1-0226

TITLE: Evaluation of Molecular Inhibitors of the c-Myc
Oncoprotein

PRINCIPAL INVESTIGATOR: Edward V. Prochownik, M.D., Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050621 060

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE February 2005	3. REPORT TYPE AND DATES COVERED Annual (19 Jan 2004 - 18 Jan 2005)	
4. TITLE AND SUBTITLE Evaluation of Molecular Inhibitors of the c-Myc Oncoprotein			5. FUNDING NUMBERS W81XWH-04-1-0226	
6. AUTHOR(S) Edward V. Prochownik, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Children's Hospital of Pittsburgh Pittsburgh, Pennsylvania 15213 <i>E-Mail:</i> Edward.prochownik@chp.edu			8. PERFORMING ORGANIZATION * REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) One of the most common molecular abnormalities in human cancers involves over-expression of the c-Myc oncoprotein. By regulating the expression of key cellular genes, c-Myc controls a number of normal biological properties such as cell cycle entry, differentiation, growth, and the decision to die or remain viable. All of these functions require that c-Myc physically associate with another protein, Max. Example of diseases in which c-Myc deregulation occurs include breast cancer (approx. 30% of cases), colon cancer (>85%), and Burkitt's lymphoma (>98%). Of relevance to this proposal is that at least <u>25%</u> of prostate cancers are also associated with c-Myc de-regulation. Additional evidence suggests that more advanced/aggressive disease may also be more commonly associated with c-Myc over-expression. Because nearly 190,000 men in the United States alone will be diagnosed with prostate cancer this year, and over 30,000 will die, the design of drugs that inhibit c-Myc would appear to be a reasonable approach.				
14. SUBJECT TERMS Oncogenes, molecular oncology, helix-loop-zipper proteins, molecular therapy				15. NUMBER OF PAGES 9
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover	1
SF 298	2
Introduction	4
Body	4
Key Research Accomplishments	6
Reportable Outcomes	8
Conclusions	8
References	9
Appendices	

Introduction

Myc oncoproteins are transcription factors, which have been implicated in a wide variety of naturally-occurring human cancers, including those of the colon, breast, and prostate (1). Using a high throughput yeast based system, we previously identified seven low molecular weight compounds that can prevent and/or disrupt the association between the c-Myc and its obligate heterodimerization partner, Max (2). Furthermore, we have demonstrated a high degree of specificity for these compounds, as evidenced by their inability to promote the dissociation of other heterodimeric transcription factors (2). In all seven cases, these compounds inhibited the *in vitro* growth of mammalian cells that express c-Myc, but not of c-Myc *-/-* cells. Finally short-term, *in vitro* treatment of c-Myc-transformed fibroblasts with several of these compounds did not affect their viability but did reduce by >90% their subsequent ability to produce tumors in nude mice without any further treatment. The significant ant-tumor effects of these agents suggest that they, or related compounds, may be effective clinical agents.

Body

Members of the Myc oncoprotein family are basic-helix-loop-helix-leucine zipper (bHLH-ZIP) transcription factors that are over-expressed in a wide variety of human cancers (1). Specifically, c-Myc de-regulation is quite frequent in prostate cancer, occurring in as many as 50% of tumors, particularly those of advanced stage (1,3). Recent evidence indicates that even short-term inhibition of c-Myc is sufficient to induce tumor regression in experimental animal models (4). We have developed a rapid, high throughput, yeast-based assay designed to identify low molecular weight compounds that inhibit and/or prevent the c-Myc-Max interaction (Fig. 1).

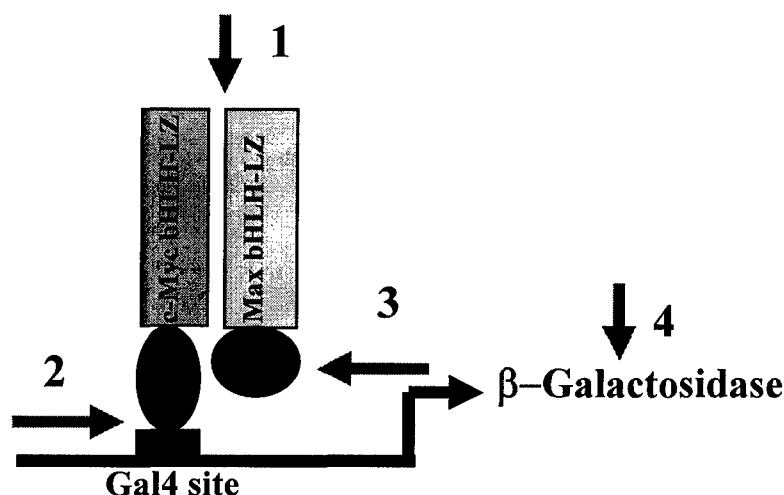


Fig. 1. Basis of the yeast 2-hybrid assay used to identify low molecular weight compounds that prevent/disrupt the c-Myc-Max interaction. The c-Myc bHLH-ZIP dimerization domain was fused with the DNA binding domain of the yeast Gal4 transcription factor whereas the Max bHLH-ZIP domain was fused with the Gal4 transactivation domain. Plasmids encoding both proteins were stably expressed in a yeast strain harboring a β -galactosidase gene with a Gal4 binding site in its promoter. c-Myc-Max association reconstitutes an active Gal4 transcription factor that induces β -galactosidase. Compounds that specifically interfered with c-Myc-Max heterodimerization (red arrow 1) prevented the induction of β -galactosidase. However, compounds that act non-specifically by preventing DNA binding or transactivation (arrows 2 and 3), or that interfere with β -galactosidase (arrow 4) would also register in this assay. To control for these non-specific effects, >30 additional yeast strains were created in which HLH and/or ZIP domains of other heterodimeric transcription factors were tested against each compound. All seven compounds showed >90% specificity for c-Myc-Max when tested against these other yeast strains (ref. 2).

We screened a chemical library of approximately 10,000 low molecular weight compounds (Chembridge, San Diego, CA) and identified 7 that demonstrate a high degree of c-Myc-Max specificity with little effect on any of >30 other heterodimeric transcription factors (Fig. 2). These compounds inhibited the *in vitro* growth of mammalian cells expressing either normal or elevated levels of c-Myc, but did not inhibit the growth of c-Myc -/- "knockout" cells (ref. 2). In our original application, we proposed to extend this work by proposing four specific tasks:

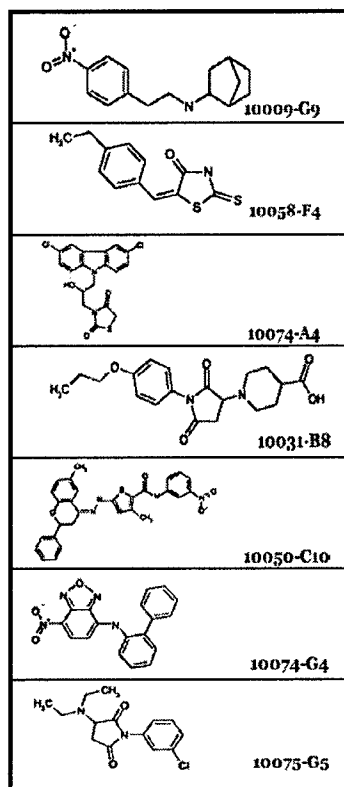


Fig. 2. Structures of the seven low molecular weight compounds that prevent/disrupt the c-Myc-Max heterodimer (ref. 2).

Statement of original tasks

Task 1: to demonstrate directly that each of the previously identified compounds either prevents or disrupts c-Myc-Max heterodimerization.

Task 2: to conduct a series of *in vivo* studies aimed at determining whether these compounds can be effectively employed to treat c-Myc over-expressing tumors.

Task 3: to determine whether any of the compounds can be utilized in combination as a means of reducing toxicity and enhancing the antineoplastic effect.

Task 4: to employ computerized "data mining" techniques to determine whether other, as yet untested, but structurally related compounds might be better suited as potential therapeutic agents.

Key Research Accomplishments

Summary

- We have determined how best to formulate several of the originally identified compounds and have begun to establish maximal tolerated doses (MTDs) for each (**Task 2**).
- We have shown that two of the original compounds work synergistically *in vitro* to inhibit the growth of c-Myc-transformed cells (**Task 3**).
- Using an *in silico* screening approach, we have identified a large number of analogs of several of our low molecular weight compounds that will be tested for improved efficacy *in vitro* and *in vivo* (**Task 4**).

Accomplishment Details

Formulation and MTDs of c-Myc-Max compounds.

Because none of the compounds shown in Fig. 2 had ever been evaluated *in vivo*, it was necessary to establish conditions that would allow for their safe and effective administration. We reasoned that this would be the first step in determining whether they could be used effectively as anti-neoplastic agents. Therefore, in collaboration with Dr. Julie Eiseman, we have established optimal conditions for formulation of some of these compounds and have begun to determine their MTDs. We established that compounds 10005-C10, 10009-G9, and 10074-A4 could be formulated in 50% hydroxypropyl- β -cyclodextrin (HPBCD) but generally not in saline, 10% DMSO-saline, or ethanol:cremophor:saline (1:1:6).

Preliminary *in vivo* experiments using several HPBCD-formulated compounds have been performed. C.B-17 SCID mice were injected intravenously with several different concentrations of #10005-C10, #10009-G9, and #100058-F4. At a concentration of 5 mg/Kg, mice receiving the first compound were found to be moribund at 24 hr and were sacrificed. Mice receiving the second compound at 13.1 mg/Kg died within 2 hr but remained alive for 14 days after receiving a mg/Kg dose. Finally, mice receiving the third compound at 7.1 mg/Kg died within 8-16 hr of dosing.

Taken together, these results indicate that three of the more potent compounds could be formulated in a standard vehicle and that rough estimates of MTDs could be determined.

Synergistic Activity of c-Myc-max compounds.

The idea that c-Myc-Max compounds might act synergistically was initially suggested by the finding that inhibition of non-Myc-Max HLH, ZIP, or HLH-ZIP heterodimers, while generally not observed, was occasionally seen, and that the spectrum of inhibition of the >30 combinations tested was unique to each compound. This suggested that each compound might be targeting a different region of the fairly large ca. 70 amino acid interface over which the c-Myc-Max interaction is known to occur. This indeed proved to be the case. As seen in Fig. 3, and as previously described, growth inhibition of the tumorigenic Rat1a-c-Myc cell line was seen with high concentrations of the compounds 10050-C10 and 10074-A4 (35 μ M and 40 μ M, respectively). Lower concentrations of these compounds, however (20 μ M 10050-C10 and 10 μ M 10074-A4) were significantly less effective. In contrast, the combination of 20 μ M 10050-C10 and 10 μ M 10074-A4 resulted in a >98% inhibition of growth. It is noteworthy that these compounds, while nearly completely inhibiting cell growth, did not result in any significant induction of apoptosis, even at the highest concentrations tested (not shown).

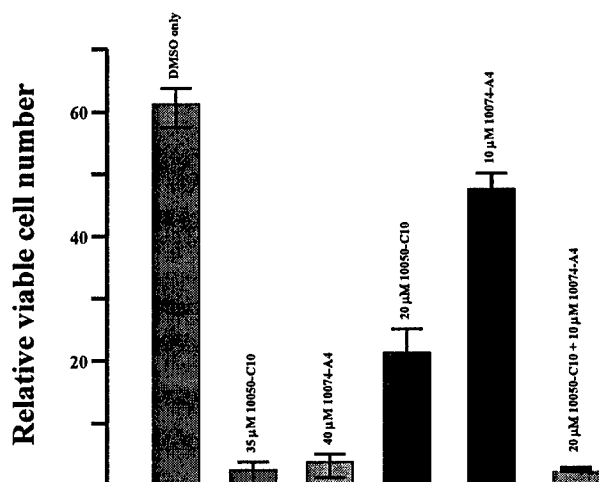


Fig. 3. Synergy of c-Myc-Max compounds. Rat1a-c-Myc cells were seeded into 24 well plates at 2,000 cells/well and allowed to attach and achieve log phase growth for 24 hr. At this time, triplicate plates were trypsinized and counted and the average cell number was arbitrarily set at 1. C-Myc-Max compounds, formulated in DMSO were then added to the indicated final concentrations. Control wells received the DMSO vehicle only. Cell numbers were again counted in triplicate on day 4 and are expressed relative to the 60.7-fold increase in control cell number. Bars represent standard errors.

Taken together, our results indicate that the compounds 10050-C10 and 10074-A4 exert a synergistic effect on the growth of tumorigenic Rat-1a-c-Myc cells.

In Silico Screening for c-Myc-Max Analogs.

Using a computer-assisted algorithm (ChemFinder Ultra 8.0, CambridgeSoft Corp. Cambridge, MA), we utilized the core structural elements of the seven compounds in an *in silico* search of another low molecular weight compound library (Chem Diversity, Cambridge, MA—5040 compounds in total) for structural analogs. In the case of compound 10058-F4, searching with the core structure shown in Fig. 4, identified a total of 10 analogs, three of which are shown.

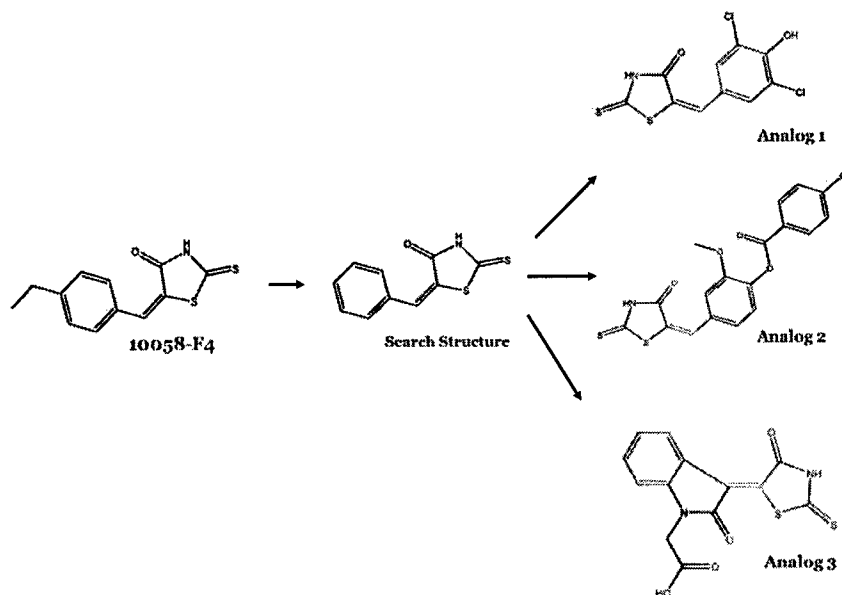


Fig. 4. *In silico* identification of compounds related to 10058-F4. The parent compound (left) was used to derive a core "search structure" depicted in the middle of the diagram. This was then used to search a library of 5040 low molecular weight compounds (Chem Diversity, Cambridge MA). A total of 10 "hits" were obtained nine of which contained the same basic 2 ring structure outlined in red. Three of these analogs are shown here (right). Screening in a similar manner for the remaining six compounds depicted in Fig. 2 identified a total of 125 analogs.

Plans for the Next Grant Period.

Continued characterization c-Myc-Max compound pharmacology.

Having developed rough estimates of the MTDs for several of the lead compounds, additional studies will be carried out to more precisely define these doses. We will conduct pharmacokinetic and efficacy studies of the most promising compounds. Initial efforts will focus upon 10058-F4 and 10074-A4, as they have shown to act synergistically *in vivo* (Fig. 3). We will determine the stability of the compounds in relevant biological matrices at 37°C and 4°C to determine appropriate handling and storage conditions. We will determine the extent of protein binding of the compounds using Amicon Centri-free centrifuge tubes or dialysis membranes (5). We will conduct a pharmacokinetic studies in Balb/c mice to characterize the plasma-concentration times time profile of each compound (and any metabolites) following i.v. administration at the maximum tolerated doses, which will have been determined based on their solubility and toxicity to mice as a single i.v. bolus (6). We will perform a standard mathematical analysis of the plasma concentration times time data using both non-compartmental and compartmental analyses to calculate the half-life values, plasma clearance and Area Under the Curve (AUC) (6-8). Cumulative urinary excretion will be determined in selected animals for determination of urinary clearance and identification of excreted metabolites. If plasma concentrations can be achieved that are similar to those that inhibit the growth of c-Myc expressing cells *in vitro*, additional studies will be performed to assess tissue distribution of the compounds. Additional pharmacokinetic studies will be performed using oral and i.p. dosing in order to determine the bioavailability of the drug by these routes (9-11). Efficacy studies will be performed using the optimum dosing schedule of the compounds based on the results of the pharmacokinetic studies (12-14). The efficacy studies will be conducted in C.B-17 SCID mice bearing c-Myc over-expressing tumors, such as those formed by Rat1a-c-Myc fibroblasts.

In Silico Screening for c-Myc-Max Analogs.

We have identified a large number of compounds which are related to our original seven lead structures (see Fig. 4 for an example). We plan to utilize the c-Myc-Max yeast two-hybrid assay depicted in Fig. 1 to evaluate the efficacy (or lack thereof) of these newly identified analogs. We anticipate that useful information will be provided both by the compounds which show activity in this assay as well as those which do not. For example, general structural features which determine overall efficacy and potency, or whose absence adversely affects these properties, should become apparent. If necessary, more refined, secondary searches, using the basic structure(s) of the most potent compounds, will also be carried out. Because the time required to screen these compounds in yeast is relatively short, we expect that their efficacies should be established within the next granting period. We further anticipate that it should be possible to evaluate the most promising compounds in c-myc-over-expressing mammalian cells *in vitro*.

Reportable Outcomes

U.S.A. patent pending: Prochownik et al; Application No: 10/459,769 (PHARMACOLOGIC INHIBITION OF MYC FUNCTION)
Filed: June 12, 2003

Conclusions

Using a yeast two-hybrid assay based approach, we have identified seven low molecular weight compounds that are capable of preventing/disrupting the association between the c-Myc oncoprotein and its obligate heterodimerization partner Max. The efficacy of these compounds has been extended to mammalian model systems both *in vitro* and *in vivo*. In the previous granting period, we have begun to determine critical pharmacologic parameters that will be essential for the eventual utilization of these compounds in pre-clinical and clinical trials. We have demonstrated that at least two of these compounds also work synergistically, suggesting that they may be affecting the c-myc-max dimerization interface at different sites. Finally, *in silico* screening of a large library of low molecular weight compounds has a large number of structural analogs whose efficacy in yeast and mammalian systems will be assessed over the next year.

References

1. Nesbit, C.E., Tersak, J.M. and Prochownik, E. V. MYC oncogenes and human neoplastic disease. *Oncogene*, 1999. **18**: 3004-3016.
2. Yin, X., Giap, C., Lazo, J.S., Prochownik, E.V. Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene*. 2003: **22**. 6151-6159
3. <http://www.myc-cancer-gene.org/>
4. Jain, M. et al. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science*. 2002. **297**: 102-4.
5. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976. **72**: 248-254.
6. Akaike, H. A new look at the statistical model identification. *IEEE Trans. Autom. Control*. 1974. **19**: 716-719.
7. D'Argenio, D.Z. and Schumitzky, A. A program package for simulation and parameter estimation in pharmacokinetic systems. *Comp. Programs Biomed*. 1979. **9**: 115-134.
8. Rocci, M.L. and Jusko, W.J. LAGRAN program for area and moments in pharmacokinetic analysis. *Comp Programs Biomed*, 1983. **16**: 203-16.
9. Egorin, M.J. et al Pharmacokinetic, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC707545) in CD2F1 mice and Fischer 344 rats. *Cancer Chemother. Pharmacol*. 2002. **49**: 7-19.
10. Egorin, M.J. et al In vitro metabolism by mouse and human liver preparations of halomon, an antitumor halogenated monoterpene. *Cancer Chemother. Pharmacol*. 1997. **41**: 9-14.
11. Egorin, M.J. et al. Metabolism of 17-(allylamino)- 17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. *Cancer Res*. 1998. **58**: 2385-2396.
12. Egorin, M.J. et al. Plasma pharmacokinetics and tissue distribution of 17-(allylamino)- 17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice. *Cancer Chemother. Pharmacol*. 2001.**47**: 291-302.
13. Eiseman J.L. et al. Tumor targeted apoptosis by a novel spermine analog 1,12-diaziridinyl-4,9-diazadodecane, results in therapeutic efficacy and enhanced radiosensitivity of human prostate cancer. *Cancer Res*. 1998. **58**:
14. Morton, C.L. et al Activation of CPT-11 in mice: identification and analysis of a highly effective plasma esterase. *Cancer Res*. 2000. **60**: 4206-4210.